RESEARCH

Clonal Selection for Lignin and Etherified Ferulates in Three Perennial Grasses

M. D. Casler,* H. G. Jung, and W. K. Coblentz

ABSTRACT

Decreased lignin concentration or decreased ferulate cross-linking between arabinoxylans and lignin are two mechanisms to increase cell-wall digestibility in plants. The objectives of this study were (i) to determine the consistency and clonal repeatability of lignin and etherified ferulates across multiple harvest dates and years, (ii) to determine if the genetic correlation between lignin and etherified ferulates can be altered by intensive selection, and (iii) to determine the effects of lignin and ferulates on digestibility of neutral detergent fiber (NDF). Thirty clones each of smooth bromegrass (*Bromus inermis* Leyss), orchardgrass (*Dactylis glomerata* L.), and reed canarygrass (*Phalaris arundinacea* L.) were evaluated in a replicated field study at four growth stages in 2004 and 2005 for NDF, lignin, esterified and etherified ferulates, and 24- and 96-h in vitro NDF digestibility (IVNDFD). Clonal means were generally repeatable across years and harvest dates. Divergent selection created clonal groups with differential lignin and etherified ferulates, but the positive correlation between these two traits was reduced only in smooth bromegrass. Both lignin and etherified ferulates were negatively correlated with 96-h IVNDFD and these relationships were maintained as all three grasses matured. Concentration of NDF was highly correlated with etherified ferulates, making it difficult to partition the impact of components of lignification on IVNDFD.

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Abbreviations: IVNDFD, in vitro neutral detergent fiber digestibility; ND, neutral detergent; NDF, neutral detergent fiber; NIRS, near-infrared reflectance spectroscopy; SEP, standard error of prediction.

Livestock performance can be improved by increasing digestibility of feeds, one of the key elements of feed quality. Digestibility of ruminant feedstocks can be increased by selection and breeding using in vitro digestibility tests, which are rapid, repeatable, and amenable to use of small sample sizes (Casler, 2001; Fahey and Hussein, 1999). Genetic improvements of in vitro digestibility have led to gains in livestock performance in a number of perennial forage crops, measured in both experiment station–based and on-farm grazing trials (Casler and Vogel, 1999).

Most early selection efforts to improve digestibility utilized some form of selection for increased dry matter or organic matter digestibility, either in vitro or in situ, the latter utilizing nylon bags placed inside the rumen of a cannulated cow (Casler, 2001). Because these measures of digestibility are defined only in terms of an interaction of rumen microbes and their enzymes with plant tissue samples suspended in buffered rumen fluid or within the rumen, measures of digestibility are not plant traits per se. While numerous studies have described quantitative inheritance, heritability, and realized selection

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gains for measures of digestibility (reviewed by Buxton and Casler, 1993; Casler and Vogel, 1999; Casler, 2001), these selection methodologies were always acting on fundamental plant traits that are correlated with digestibility.

Mechanisms for increased dry matter or organic matter digestibility include reduced cell-wall or fiber concentration, increased water-soluble carbohydrate concentration, modified lignin composition, decreased lignin concentration, or decreased ferulate cross-linking between arabinoxylans and lignin (Casler, 2001; Vogel and Jung, 2001). Because soluble carbohydrates form the polysaccharide pool from which structural carbohydrates are synthesized, the first two mechanisms may be closely related or nearly identical to each other. Consistently negative correlations between water-soluble carbohydrates and neutral detergent fiber (NDF) (Lee et al., 2001; Radojevic et al., 1994) suggest that the soluble and structural carbohydrate pool sizes are inversely related to each other, as would be expected if the structural pool draws substrate from the soluble pool.

In recent years interest has heightened in the use of in vitro digestibility methods that are focused specifically on cell-wall or fiber digestibility, with NDF as the leading subject of these efforts (Casler and Jung, 2006; Jung and Lamb, 2006). Mechanisms for increased NDF digestibility include modified lignin composition, decreased lignin concentration, or decreased ferulate cross-linking between arabinoxylans and lignin (Casler, 2001; Jung and Deetz, 1993; Vogel and Jung, 2001). Ferulates are esterified to α-L-arabinose side-chains of xylans and etherified to lignin (Jung, 2003; MacAdam and Grabber, 2002; Ralph et al., 1995). This oxidative coupling of ferulates appears to serve multiple functions within plants, including cell-wall stiffening, cessation of growth, and pest resistance (Grabber, 2005) and it also may serve as an antiherbivory mechanism (Falkner and Casler, 1998).

As plants mature, large-scale changes to the cell wall are often associated closely with changes in reproductive maturity, as larger tillers, taller plants, and seed development all require greater cell-wall accumulation to maintain plant biomass in an upright position up to 2 m above the ground. One hypothesis to explain the large amount of genetic variability for cell-wall composition, a large proportion of which is independent of the timing of reproductive maturity (Casler, 2001), is the partial decoupling of cell-wall synthesis from reproductive maturity (Casler and Hatfield, 2006; Jung and Lamb, 2006). Cell-wall development and reproductive maturation are processes that inexorably advance toward the evolutionary goal of sexual reproduction, but not necessarily at the same rate and with the same result in all genotypes. Furthermore, phenotypic correlations between lignin and etherified ferulates ranging from $r = 0.24$ to 0.65 (Casler and Jung, 2006) suggest that the various processes leading to secondary wall development and cessation of cell growth, are not synchronized consistently in all genotypes. Some genotypes accumulate lignin at a faster rate, but may accumulate etherified ferulates at a slower rate. Both lignin and etherified ferulates negatively impact extent of NDF digestibility, these effects can occur independently of each other, and it appears that genotypes with high NDF digestibility may achieve that state by different mechanisms (Casler and Jung, 2006).

The objectives of this study were (i) to determine the consistency and repeatability of clonal performance for lignin and etherified ferulates across multiple harvest dates and years, (ii) to determine if the apparent genetic correlation between lignin and etherified ferulates can be altered by intensive selection in three perennial grasses, and (iii) to determine the effects of lignin and ferulates on digestibility of NDF.

MATERIALS AND METHODS

A total of 775 smooth bromegrass (*Bromus inermis* Leyss) plants, 729 reed canarygrass (*Phalaris arundinacea* L.) plants, and 280 orchardgrass (*Dactylis glomerata* L.) plants were evaluated for lignin and etherified ferulate concentration over multiple harvests of vegetative growth for 2 yr (Casler and Jung, 2006). Based on means over all harvests, 30 plants of each species were selected from this study. Seven or eight plants were chosen from within each of the following classifications: high-lignin and high-etherified ferulates, high-lignin and low-etherified ferulates, low-lignin and high-etherified ferulates, and low-lignin and low-etherified ferulates. Because the phenotypic correlation coefficients between lignin and etherified ferulates were positive ($r = 0.24$ to 0.65; Casler and Jung, 2006), some compromise was required to select plants within the two middle categories. In these cases, "high" or "low" were defined as above or below the experiment mean, respectively. Within each species, the 30 clones represented an ellipse selected from the outer borders of the bivariate distribution of lignin and etherified ferulates.

Each plant was clonally propagated and used to establish a new field experiment at Arlington, WI, on a Plano silt loam soil (fine-silty, mixed, mesic Typic Argiudoll) in May 2003. Plants were established on a 0.9-m spacing in both directions and the spaces between plants were kept weed free by application of preemergence herbicides in early spring before initiation of growth and by hand weeding. Clonal ramets consisted of approximately 6 to 10 tillers at the time of transplanting. Each species was established as a separate experiment with 12 complete blocks of 30 clones. Within each experiment the 12 blocks were arranged into a split-plot randomization in a randomized complete block design with three replicates and four harvest dates in which harvest dates were whole plots and clones were subplots.

Plants were harvested on one of four harvest dates in spring 2004 and 2005. Plants were fertilized with 112 kg N ha^{-1} in early spring of both years. Harvest dates were designed to sample plants at one of four growth stages: vegetative (~20 cm in height), jointing (two or three elongated internodes), early heading (first panicle fully emerged), and anthesis (all panicles in anthesis). Harvest dates varied widely among species and years due to inconsistent relationships between growth stages and calendar dates among species and years. All 30 clones within a block were harvested within a 15-min period on each harvest

date. There was little or no genetic variation for stage of maturity within each experiment due to photoperiodism in smooth bromegrass, to lack of genetic variability for maturity within the reed canarygrass population, or due to selection for constant heading date within the group of 30 orchardgrass clones. A random sample of 10 to 30 tillers was hand-clipped from each plant at a 9-cm cutting height.

Samples were stored in paper bags and dried at 60°C. Dry samples were ground through a 1-mm screen of a Wiley-type mill and scanned by near-infrared reflectance spectroscopy (NIRS). Cluster analysis of reflectance spectra was used to develop a subset of 84 samples for calibration development (Shenk and Westerhaus, 1991). The concentration of NDF was determined using the procedure of Van Soest et al. (1991), omitting the sodium sulfite, α-amylase, and ash-correction steps. Klason lignin concentration was measured as the ash-free residue remaining after cell-wall polysaccharide hydrolysis (Theander et al., 1995). The Klason lignin procedure was utilized to remain consistent with our previous work on selection and breeding for lignin and etherified ferulate (Casler and Jung, 1999; Jung and Casler, 1991) and because it provides a more accurate estimate of total lignin in the cell wall (Hatfield et al., 1994; Jung et al., 1999; Lowery et al., 1994). Esterified ferulate concentration in the cell wall was determined by 2 M NaOH extraction and high performance liquid chromatography analysis (Jung and Shalita-Jones, 1990). Concentration of etherified ferulate was computed as the difference between total ferulate, obtained by 4 M NaOH extraction at 170°C for 2 h, and the esterified fraction (Iiyama et al., 1990). Lignin and phenolic acids were determined on duplicate samples. In vitro digestible NDF was determined in triplicate using 24- and 96-h fermentations according to the procedure of Casler (1987). Fermentation times of 24 and 96 h were used to provide estimates of rapidly and potentially digestible NDF fractions, respectively. Means over laboratory replicates were used to develop calibration equations, which were then used to develop predicted values for all variables on all samples. Lignin and ferulates were converted from a dry-matter basis to an NDF basis after predictions were generated using NIRS. In vitro digestibility of the NDF fraction (IVNDFD) was computed from the indigestible NDF residue and total NDF as described by Casler (1987). Calibration statistics for NIRS were SEP = 12.6 g kg^{-1} and R^2 = 0.98 for NDF; SEP = 19.1 g kg^{-1} and R^2 = 0.67 for Klason lignin; SEP = 0.58 g kg^{-1} and R^2 = 0.57 for esterified ferulates; SEP = 0.96 g kg^{-1} and R^2 = 0.77 for etherified ferulates; SEP = 6.82 g kg^{-1} and R^2 = 0.28 for 24-h IVNDFD; and SEP = 3.11 g kg^{-1} and R^2 = 0.94 for 96-h IVNDFD.

Data were analyzed by analysis of variance assuming all effects to be random, except for selection groups (high vs. low lignin or etherified ferulates). Broad-sense heritability was computed using the formula $H = s^2_C/(s^2_C + s^2_{CY}/\gamma + s^2_{CH}/h + s^2_{CYH}/\gamma h + s^2_e/\gamma hr)$, where s^2_C = the estimated variance component for clones, s^2_{CY} = the estimated variance component for clone × year, s^2_{CH} = the estimated variance component for clone × harvest, s^2_{CYH} = the estimated variance component for clone × year × harvest, s^2_e = the estimated error variance component, γ = number of years, h = number of harvests, and r = number of replicates. High-lignin vs. low-lignin and high-etherified ferulates vs. low-etherified ferulates (15 clones with each group) comparisons were made using contrasts within analysis of variance. The clone × harvest interaction was partitioned into linear and nonlinear components to test differences among clones in their linear response to harvest date.

Relationships among harvest dates within each variable (i.e., the clone × harvest interaction) were investigated by Kendall's tau, the coefficient of concordance (Conover, 1971). The coefficient of concordance is analogous to a pooled rank correlation coefficient across multiple variables (harvests)—values close to 1 indicate concordance across all harvest dates, while values close to −1 indicate discordance across all harvest dates. Relationships among variables were investigated by phenotypic correlation coefficients, linear regression coefficients, or standardized partial regression coefficients (Draper and Smith, 1981). These analyses were conducted separately for each species, year, and harvest date. Because results were generally homogeneous across years and harvest dates, these results were pooled across years and harvest dates.

RESULTS AND DISCUSSION

Repeatability of Clone Means

Broad-sense heritability, equivalent to repeatability of clone means, ranged from 0.42 to 0.88, indicating that most of the variability among clone means was due to genotypic effects and that clone × year and clone × harvest date interactions were relatively unimportant (Table 1). There were two exceptions: lignin of smooth bromegrass and esterified ferulates of reed canarygrass with H < 0.5. The relatively high broad-sense heritability values indicated that clone means were generally high in repeatability across harvest dates and years, supporting previous studies of these variables in these grasses (Casler and Jung, 2006) and other species (Casler, 2001).

Despite the relatively high broad-sense heritabilities, the clone × harvest date interaction was significant for every variable measured on all three species. The partition of this interaction into linear and nonlinear components indicated that 31 to 77% of this interaction was due to differences among clones in linear response to harvest date (Table 1). Changes in these variables were linear for the duration of this experiment in both years. Deviations from linear responses were due to random or unpredictable effects and not to predictable nonlinear effects. Linear responses of individual clone means to harvest date generally had high R^2 values, with the vast majority of $R^2 > 0.75$. The most notable exceptions to this were esterified ferulates of orchardgrass and smooth bromegrass and lignin of orchardgrass.

The high repeatability of clone means between years was illustrated by the high phenotypic correlation coefficients between 2004 and 2005, in which all but two values were >0.5 (Table 1). Linear responses of clone means to harvest date were considerably less repeatable between years for all variables, perhaps due to the complexity of a second-order statistic such as a linear regression slope. Rates of change with maturation are generally estimated

Table 1. Summary statistics for six variables measured on three perennial grass species, including broad-sense heritability (H), the percentage of the clone × harvest date interactions (C × D) associated with differences among clones in linear responses to date, the percentage of individual-clone regressions on date with $R^2 > 0.75$, and phenotypic correlation coefficients among means and slopes of individual clones.

Species	Variable†	H	C × D linear	$R^2 > 0.75$	Phenotypic correlation coefficients			
					Mean2004 vs. Mean2005	Slope2004 vs. Slope2005	Mean2004 vs. Slope2004	Mean2005 vs. Slope2005
			%					
Smooth bromegrass	NDF	0.81	36**	82	0.81**	0.44**	0.50**	0.37*
Reed canarygrass	NDF	0.81	31**	98	0.69**	–0.02	0.26	–0.08
Orchardgrass	NDF	0.84	37*	87	0.75**	–0.11	–0.12	–0.64
Smooth bromegrass	Lignin	0.47	32**	82	0.71**	0.30*	–0.06	–0.42
Reed canarygrass	Lignin	0.72	59**	65	0.69**	0.39*	0.44**	0.37*
Orchardgrass	Lignin	0.82	46**	37	0.54**	0.46**	–0.02	–0.14
Smooth bromegrass	EstFA	0.61	51**	8	0.47**	0.16	0.16	–0.04
Reed canarygrass	EstFA	0.42	65**	58	0.39*	0.69**	0.57**	–0.25
Orchardgrass	EstFA	0.59	65**	37	0.51**	0.54**	–0.11	–0.32
Smooth bromegrass	EthFA	0.87	38*	90	0.83**	0.27	0.16	0.17
Reed canarygrass	EthFA	0.83	51**	100	0.76**	0.23	0.85**	0.53**
Orchardgrass	EthFA	0.88	43**	72	0.86**	0.18	0.48**	0.06
Smooth bromegrass	24-h IVNDFD	0.76	34**	72	0.65**	0.59**	0.38*	0.52**
Reed canarygrass	24-h IVNDFD	0.67	61**	100	0.60**	0.45**	0.74**	0.40*
Orchardgrass	24-h IVNDFD	0.71	52**	63	0.78**	0.27	–0.40	–0.39
Smooth bromegrass	96-h IVNDFD	0.79	47**	72	0.77**	0.69**	0.70**	0.91**
Reed canarygrass	96-h IVNDFD	0.51	77**	87	0.66**	0.71**	0.91**	0.60**
Orchardgrass	96-h IVNDFD	0.74	71**	87	0.79**	0.46**	0.44**	0.39*

*Correlation coefficient significantly greater than zero at $P < 0.05$ (one-tailed hypothesis tests).

**Correlation coefficient significantly greater than zero at $P < 0.01$ (one-tailed hypothesis tests).

†NDF , neutral detergent fiber; EstFA , esterified ferulates; EthFA , etherified ferulates; IVNDFD = in vitro NDF digestibility (24 or 96 h).

with reduced precision relative to genotype or cultivar means and are probably subject to a wider range of environmental effects than simple genotype means. There was evidence of a positive correlation between mean and slope in 8 of 18 cases (both years), indicating that there was some tendency for genotypic variability among clones to be greater at the more advance maturity stages. This was particularly true for 96-h IVNDFD.

The high repeatability of clone means across harvest dates is illustrated by the relatively high coefficients of concordance in Table 2, all values significant at $P < 0.05$ except for lignin of smooth bromegrass in 2005 and reed canarygrass in 2004. The moderate to high values in Table 2 (measured on the typical scale of any correlation coefficient) indicated that clone means were positively correlated across all harvest dates and that there were no trends for this correlation to decay with increasing time between the more temporally distant harvest dates. Rank correlations between harvest dates were generally homogeneous so that the coefficients of concordance in Table 2 were very similar to the average rank correlation between harvest dates.

Relationship between Lignin and Etherified Ferulates

One of our long-term goals is to develop a highly selected group of clones in which the positive correlation between

Table 2. Coefficients of concordance, measuring the consistency of rankings of 30 clone means across four harvest dates.

Variable	Smooth bromegrass 2004	Smooth bromegrass 2005	Reed canarygrass 2004	Reed canarygrass 2005	Orchardgrass 2004	Orchardgrass 2005
Neutral detergent fiber (NDF)	0.63**	0.87**	0.71**	0.78**	0.68**	0.71**
Klason lignin	0.49**	0.24	0.25	0.53**	0.63**	0.56**
Esterified ferulates	0.63**	0.42*	0.39**	0.61**	0.64**	0.41**
Etherified ferulates	0.62**	0.68**	0.67**	0.72**	0.67**	0.60**
24-h in vitro NDF digestibility	0.51**	0.57**	0.58**	0.67**	0.60**	0.64**
96-h in vitro NDF digestibility	0.73**	0.40*	0.47**	0.58**	0.56**	0.62**

*Coefficient of concordance significantly different from zero at $P < 0.05$.

**Coefficient of concordance significantly different from zero at $P < 0.01$.

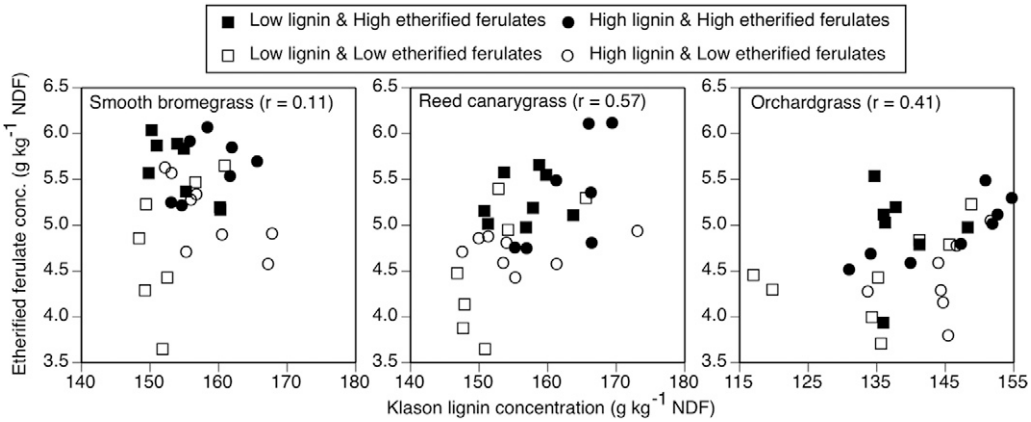

Figure 1. Scatterplot of the relationship between Klason lignin concentration and etherified ferulate concentration for four groups of selected clones within each of three perennial grass species. NDF, neutral detergent fiber.

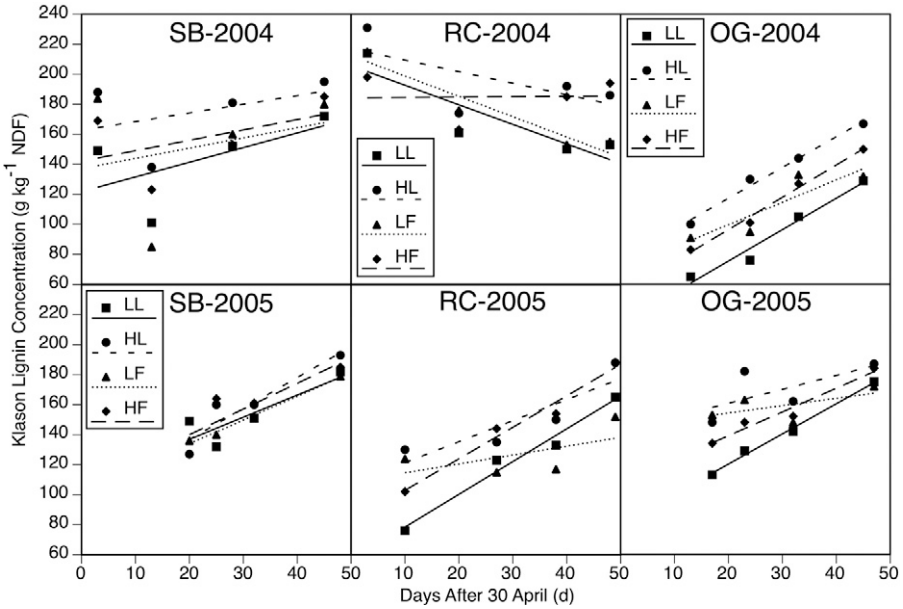

Figure 2. Linear responses of Klason lignin concentration to harvest date for high-lignin (HL), low-lignin (LL), high-etherified ferulate (HF), and low-etherified ferulate (LF) clones selected within each of three perennial grass species (slopes and R^2 values are shown in Table 3). NDF, neutral detergent fiber.

lignin and etherified ferulates has either been broken or significantly reduced in magnitude. These clones would be used to test very specific hypotheses about the independent effects of lignin and etherified ferulates on ruminant nutrition, rumen function, animal performance, and plant fitness. We earlier reported results that suggested this goal would be possible, using repeated or multistage selection of individual clones of smooth bromegrass (Casler and Jung, 1999). Our current results for smooth bromegrass confirm those earlier results, that the phenotypic correlation has been reduced from 0.41 (Casler and Jung, 2006) to 0.11 (Fig. 1). However, for the other two species, there was little change in the phenotypic correlation between these two traits, suggesting that a second stage of clonal selection, among these two groups of 30 clones, will be required to create smaller groups of clones that will allow independent comparisons of the type made by Casler and Jung (1999). The scatter of points in Fig. 1 illustrates both positive and negative results from the clonal selection process. On the negative side, the original clonal classifications were erroneous in some cases, illustrating a classic problem with selection for quantitative traits that are subject to considerable environmental and laboratory variation. While clonal repeatability is high on samples collected within an experimental nursery, repeatability is reduced when the clones are transplanted to a new experiment, likely reducing both broad- and narrow-sense heritability in selection for lignin or etherified ferulates. On the positive side, the four selection groups, taken as a whole, behaved as predicted by selection, with a general trend toward their respective corner of each scatterplot in Fig. 1. These results suggest that some clones have greater inherent stability and/or repeatability of both lignin and etherified ferulate concentrations, making them excellent candidates to pass the next stage of selection toward development of our desired set of phenotypically divergent clones.

Linear regressions on harvest date are illustrated in Fig. 2 to 4 and Table 3 for four clones, the two clones with the greatest mean difference in lignin and the two clones with the greatest mean difference in etherified ferulates. The unexpectedly low lignin values for the second harvest (jointing) of smooth bromegrass and reed canarygrass in 2004 (Fig. 2) illustrates a typical reason for unpredictable lack-of-fit to the linear regression responses. In both of these cases, these responses were due to linear increases of NDF concentration across all four harvest dates, but a delay in increase of lignification, on a dry matter basis, until sometime after jointing, i.e., no change in dry-matter lignin (lignin expressed on a dry-matter basis) between vegetative and jointing growth stages. There were a number of crossover interactions for lignin illustrated in Fig. 2, largely due to a relatively small number of clones with relatively flat, nonsignificant linear regressions. However, as pointed out in Table 1 and observable in Fig. 2, these were not necessarily consistent between years. An additional inconsistency was observed for reed

canarygrass in 2004, for which there was a general decline in lignin concentration, due to a faster increase in NDF than in dry-matter lignin across harvest dates.

The more consistent linearity and the greater concordance of etherified ferulates across harvest dates, compared to that observed for lignin, can be observed in Fig. 3 and Table 3. Etherified ferulates increased for all clones of each species in each year. The positive correlation between mean and slope of etherified ferulates of reed canarygrass is illustrated in Fig. 3, demonstrating the increase in genetic variability with advancing maturity in both years. Compared to lignin, these results suggest that it should be simpler and more effective to make genetic improvements in etherified ferulates, largely due to their greater consistency across harvest dates and years.

Divergent clonal selection for lignin or etherified ferulates resulted in an expansion of genotypic variability for 96-h IVNDFD with advancing maturity of orchardgrass and reed canarygrass, and less so for smooth bromegrass (Fig. 4, Table 3). This effect was by far strongest for etherified ferulates; the low-etherified ferulate clone always had the least response of 96-h IVNDFD to advancing maturity while the high-etherified ferulate clone had the greatest response of 96-h IVNDFD to advancing maturity in all cases except smooth bromegrass in 2005 (Table 3). For orchardgrass and reed canarygrass, this response was strikingly obvious (Fig. 4) and suggested that selection for low-etherified ferulates might be a mechanism to drastically reduce the loss of digestibility with advancing maturity. A definite conclusion regarding this hypothesis cannot be drawn, because these analyses were based on whole-plant samples. The relative ratios of plant parts changes dramatically across these four growth stages and may be responsible for most or all of the expansion of genotypic variability in 96-h IVNDFD (Casler, 1999b). Alternatively, if chemical composition is highly correlated across plant

Table 3. Linear responses of lignin, etherified ferulates (EthFA), and 96-h in vitro neutral detergent fiber digestibility (IVNDFD) to harvest date during spring growth of 24 selected clones of three temperate perennial grass species, corresponding to the regressions shown in Fig. 2 to 4.

Species and year	Selection criterion	Klason lignin		Etherified ferulates		96-h IVNDFD	
		Slope	R^2	Slope	R^2	Slope	R^2
Smooth bromegrass		g kg^{-1} NDF d^{-1}		g kg^{-1} NDF d^{-1}		g kg^{-1} NDF d^{-1}	
2004	Low lignin	0.97	0.35	0.101	0.94	–7.82	1.00
2004	High lignin	0.58	0.17	0.080	0.90	–7.54	0.99
2004	Low EthFA	0.68	0.07	0.096	0.83	–7.09	0.99
2004	High EthFA	0.69	0.23	0.112	0.99	–8.03	0.99
2005	Low lignin	1.47	0.75	0.158	0.83	–9.65	0.95
2005	High lignin	2.06	0.88	0.156	0.78	–12.14	0.97
2005	Low EthFA	1.57	0.99	0.139	0.71	–9.37	0.98
2005	High EthFA	1.70	0.76	0.159	0.73	–10.89	0.96
Reed canarygrass							
2004	Low lignin	–1.32	0.77	0.111	0.93	–6.06	0.97
2004	High lignin	–0.75	0.39	0.130	0.97	–8.45	0.99
2004	Low EthFA	–1.35	0.91	0.097	0.82	–5.03	0.98
2004	High EthFA	0.01	0.00	0.146	0.98	–11.10	0.99
2005	Low lignin	2.17	0.97	0.119	0.98	–4.10	0.72
2005	High lignin	1.40	0.79	0.094	0.96	–3.00	0.61
2005	Low EthFA	0.60	0.34	0.075	0.97	–0.45	0.03
2005	High EthFA	2.09	0.98	0.127	1.00	–6.50	0.99
Orchardgrass							
2004	Low lignin	2.09	0.97	0.094	0.94	–2.47	0.73
2004	High lignin	2.04	0.99	0.150	0.95	–5.11	0.81
2004	Low EthFA	1.50	0.78	0.115	0.87	0.26	0.05
2004	High EthFA	2.15	0.99	0.121	0.95	–6.07	0.88
2005	Low lignin	2.02	0.99	0.119	0.92	–8.69	0.93
2005	High lignin	0.94	0.45	0.116	0.94	–8.98	0.93
2005	Low EthFA	0.49	0.36	0.126	0.94	–5.38	0.82
2005	High EthFA	1.57	0.96	0.162	0.97	–10.85	0.89

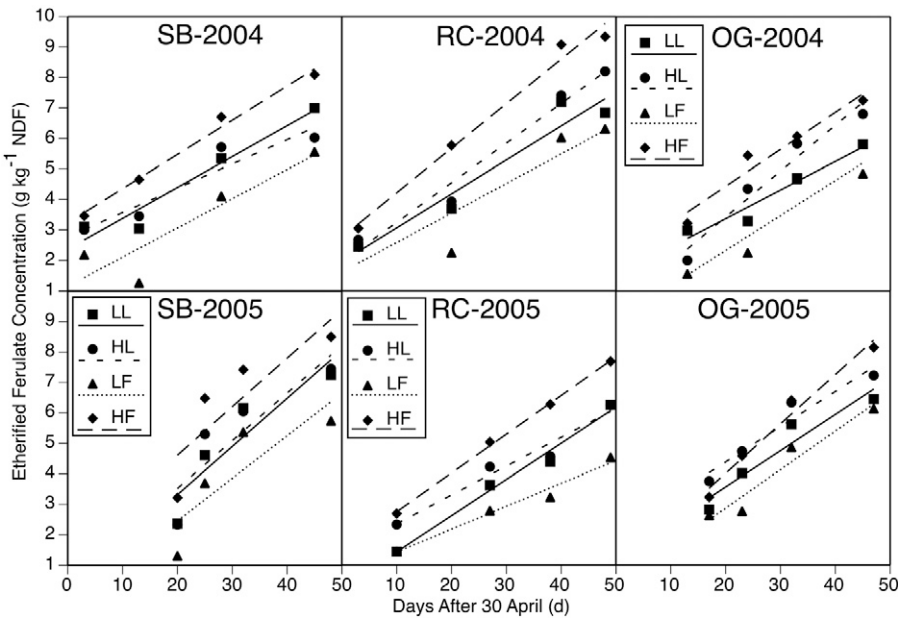

Figure 3. Linear responses of etherified ferulates concentration to harvest date for high-lignin (HL), low-lignin (LL), high-etherified ferulate (HF), and low-etherified ferulate (LF) clones selected within each of three perennial grass species (slopes and R^2 values are shown in Table 3). NDF, neutral detergent fiber; SB, smooth bromegrass; RC, reed canarygrass; OG, orchardgrass.

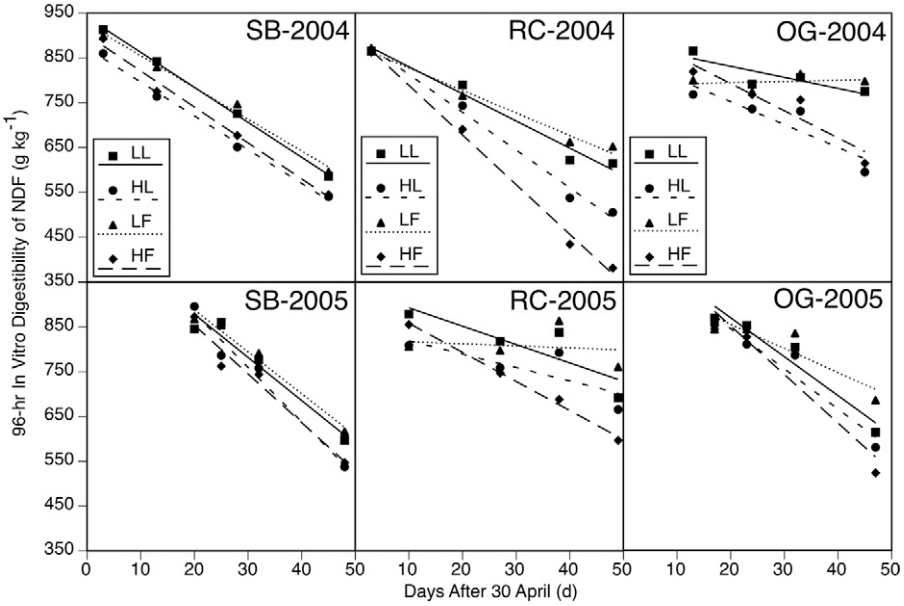

Figure 4. Linear responses of 96-h in vitro neutral detergent fiber digestibility to harvest date for high-lignin (HL), low-lignin (LL), high-etherified ferulate (HF), and low-etherified ferulate (LF) clones selected within each of three perennial grass species (slopes and R^2 values are shown in Table 3). NDF, neutral detergent fiber; SB, smooth bromegrass; RC, reed canarygrass; OG, orchardgrass.

parts, as observed for NDF (Casler, 1999b), these results suggest that selection may be more efficient based on stem samples harvested at a more advanced stage of maturity.

Divergent selection for lignin resulted in clonal groups that differed in lignin concentration by 2.1 to 5.9% of the experiment mean (Table 4). Clonal divergence was greatest in orchardgrass, the species with the lowest selection intensity (5.3 compared to 1.9 or 2.0%). Divergent selection led to small and inconsistent changes in NDF concentration: no change for smooth bromegrass, a positive response for reed canarygrass, and a negative response for orchardgrass. A positive association with lignin was observed for etherified ferulates of smooth bromegrass and reed canarygrass, as expected based on the positive correlation between lignin and etherified ferulates observed in all three of the original populations Casler and Jung (2006). These two species had the strongest correlation between lignin and etherified ferulates in the original populations ($r = 0.41$ for smooth bromegrass, $r = 0.65$ for reed canarygrass, and $r = 0.24$ for orchardgrass). Divergent clonal selection for lignin resulted in small negative associations with IVNDFD in all cases except 24-h IVNDFD of orchardgrass. This apparent anomaly was likely due to the negative association of lignin with NDF, which led to greater 24-h IVNDFD in the high-lignin clones. We have previously shown that NDF is more strongly correlated with 24-h IVNDFD than either lignin or etherified ferulates (Casler and Jung, 2006).

Divergent selection for etherified ferulates was far more effective than selection for lignin, resulting in clonal groups that differed in etherified ferulates by 9.6 to 10.5% of the experiment mean (Table 5). As predicted by Casler and Jung (2006), selection for low etherified ferulates resulted in plants with reduced NDF concentration, with divergence in NDF of 2.8 to 4.9% of the mean. Low-etherified ferulate clones of reed canarygrass and orchardgrass also had reduced lignin concentration. The large positive correlated changes in etherified ferulates, lignin, and NDF led to a large negative effect on both 24- and 96-h IVNDFD for all three species.

Table 4. Means of high-lignin vs. low-lignin groups of selected clones within each of three temperate perennial grass species.†

Species and selection group	NDF	Lignin	Esterified ferulates	Etherified ferulates	24-h I VNDFD	96-h IVNDFD
	g kg^{-1}			g kg^{-1} NDF		
Smooth bromegrass						
High lignin	518	158	4.36	5.40	245	742
Low lignin	519	154	4.38	5.25	248	750
Difference, %‡	–0.2	2.1	–0.4	2.9	–1.3	–1.0
P value	0.7711	0.0054	0.6587	0.0081	<0.0001	<0.0001
Reed canarygrass						
High lignin	538	161	5.61	5.13	266	709
Low lignin	529	153	5.65	4.82	274	733
Difference, %‡	1.7	4.8	–0.7	6.1	–3.2	–3.3
P value	0.0134	<0.0001	0.4534	0.0006	<0.0001	<0.0001
Orchardgrass						
High lignin	515	145	4.94	4.70	284	769
Low lignin	525	137	4.97	4.69	281	775
Difference, %‡	–2.0	5.9	–0.6	0.1	1.1	–0.9
P value	0.0003	<0.0001	0.4588	0.8995	<0.0001	<0.0001

†IVNDFD, in vitro neutral detergent fiber digestibility; NDF, neutral detergent fiber.

‡Percentage of the mean within each species.

Relationship of Lignin and Etherified Ferulates to NDF Digestibility

Because of these correlations, simple contrast analyses, such as those reported in Tables 4 and 5, could not specifically assign divergence in digestibility to the selection criteria, lignin or etherified ferulate concentration. Lignin and etherified ferulates had negative effects on 96-h IVNDFD, with both the slope and fit of these relationships varying among species (Fig. 5, Table 6). Because esterified and etherified ferulates are negatively correlated with each other (Jung and

Casler, 1991), esterified ferulates had a positive effect on 96-h IVNDFD, although the strong intercorrelations among the two types of ferulates and lignin prevents any type of cause and effect to be assigned from these analyses. Evidence that lignin deposition initiates through attachment of monolignols to ferulate esters of arabinoxylan, with lignin polymer growth proceeding from this initial complex (Ralph et al., 1995), suggests strong coordination of cell wall development and lignification. This complexity makes it difficult to partition the effects of specific cell-wall components on digestibility.

The standardized partial least squares method was utilized to estimate independent effects of lignin and ferulates on IVNDFD (Table 7). In contrast to observations made on the original populations before selection, lignin had no effect on 24-h IVNDFD, while etherified ferulates had a negative effect on 24-h IVNDFD for the highly selected clones of all three species. Selection has apparently altered the relationship of 24-h IVNDFD with these components of the cell wall. This may have been a result of the strong and positive correlations between NDF and etherified ferulates (Table 5). Etherified ferulates and NDF were positively correlated in the original populations (r = 0.59 to 0.81; Casler and Jung, 2006) and among the selected clones (r = 0.56 to 0.83, all with $P < 0.01$). Because NDF was much more closely associated with 24-h IVNDFD than lignin or ferulates in the original populations, it is possible that the current association of etherified ferulates with 24-h IVNDFD is due wholly or partly to its positive correlation with NDF.

Smooth bromegrass plants selected for divergent NDF concentration did not differ in cell-wall concentration (Casler and Hatfield, 2006). Rather, low-NDF plants have a cell wall that appears to be more soluble in neutral detergent (ND) solution, resulting in a cell wall with a larger ND-soluble fraction and a smaller ND-insoluble fraction. Digestion proceeds most rapidly in cell walls that are least lignified and have the least secondary wall development (Akin and Amos, 1975; Akin and Burdick, 1981), but solubility of cell walls in rumen fluid may also be an important factor influencing 24-h IVNDFD. Low-NDF plants and, by association, low-etherified ferulate plants may have higher 24-h IVNDFD because their cell walls are more susceptible to rapid solubility and fermentation of polysaccharides compared to high-NDF (and high-etherified ferulate) plants.

At 96 h, the relationships of lignin and ferulates were largely as observed in the original populations (Table 7).

Table 5. Means of high-etherified ferulate vs. low-etherified ferulate groups of selected clones within each of three temperate perennial grass species.†

Species and selection group	NDF	Lignin	Esterified ferulates	Etherified ferulates	24-h IVNDFD	96-h IVNDFD
	g kg^{-1}			g kg^{-1} NDF		
Smooth bromegrass						
High etherified ferulates	527	156	4.31	5.60	242	740
Low etherified ferulates	510	156	4.43	5.05	252	753
Difference, %‡	3.2	0.4	–2.7	10.3	–3.9	–1.7
P value	<0.0001	0.5233	0.0049	<0.0001	<0.0001	<0.0001
Reed canarygrass						
High etherified ferulates	541	161	5.63	5.22	266	706
Low etherified ferulates	526	153	5.64	4.74	274	735
Difference, %‡	2.8	4.9	–0.3	9.6	–3.1	–4.0
P value	0.0002	0.0000	0.7934	<0.0001	<0.0001	<0.0001
Orchardgrass						
High etherified ferulates	533	142	4.88	4.94	273	758
Low etherified ferulates	508	139	5.04	4.45	292	786
Difference, %‡	4.9	2.1	–3.2	10.5	–6.7	–3.6
P value	<0.0001	0.0219	0.0007	<0.0001	<0.0001	<0.0001

†IVNDFD, in vitro neutral detergent fiber digestibility; NDF, neutral detergent fiber.

‡Percentage of the mean within each species.

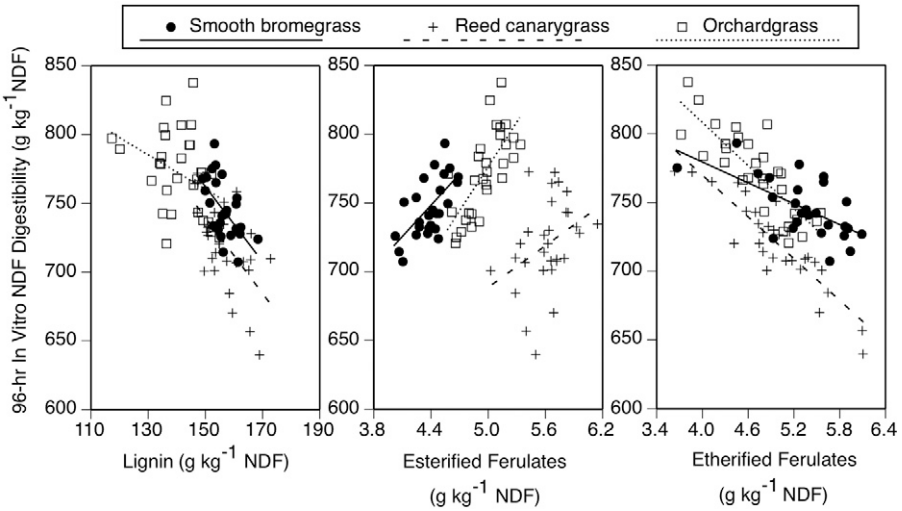

Figure 5. Linear regressions of 96-h in vitro neutral detergent fiber (NDF) digestibility on lignin, esterified ferulates, or etherified ferulates for three perennial grass species.

Table 6. Linear regressions of 96-h in vitro neutral detergent fiber (NDF) digestibility on lignin, esterified ferulates, or etherified ferulates for three perennial grass species.

Species	Klason lignin		Esterified ferulates		Etherified ferulates	
	Slope	R^2	Slope	R^2	Slope	R^2
Smooth bromegrass	–2.74 ± 0.77	0.34	76.4 ± 18.7	0.41	–25.4 ± 6.4	0.40
Reed canarygrass	–2.77 ± 0.68	0.37	50.8 ± 24.4	0.13	–50.6 ± 4.8	0.80
Orchardgrass	–1.25 ± 0.59	0.14	107.7 ± 19.4	0.52	–52.1 ± 6.6	0.69

All three components had a significant effect, negative for lignin and etherified ferulates and positive for esterified ferulates, largely as expected. Similarly to previous results (Casler and Jung, 1999, 2006), lignin and etherified ferulates had similar effects on 96-h IVNDFD, taken as a whole across the three species. It is clear from this and previous research that there are two distinct mechanisms by which 96-h NDF digestibility can be improved in perennial C3 grasses—reduced lignin concentration or reduced etherified ferulate concentration. Although these two components of the cell wall are positively correlated with each other, their effects on 96-h IVNDFD are independent, as determined by two different statistical methods—standardized partial least squares and orthogonal contrasts (Casler and Jung, 1999, 2006; Table 7 of this paper).

Table 7. Standardized partial least squares regression coefficients, standard errors, and *P* values (in parentheses) for the effects of Klason lignin and ferulates on 24- or 96-h in vitro neutral detergent fiber digestibility (IVNDFD).

Variable	Smooth bromegrass		Reed canarygrass		Orchardgrass	
24-h IVNDFD						
Klason lignin	−0.01 ± 0.06	(0.8535)	−0.09 ± 0.15	(0.5496)	−0.13 ± 0.08	(0.1347)
Esterified ferulates	0.36 ± 0.07	(0.0017)	0.12 ± 0.08	(0.1712)	0.24 ± 0.14	(0.1291)
Etherified ferulates	−0.29 ± 0.12	(0.0497)	−0.63 ± 0.11	(0.0007)	−0.33 ± 0.12	(0.0290)
Residual	0.73 ± 0.06	(<0.0001)	0.51 ± 0.08	(0.0003)	0.63 ± 0.05	(<0.0001)
96-h IVNDFD						
Klason lignin	−0.46 ± 0.03	(<0.0001)	−0.36 ± 0.13	(0.0229)	−0.26 ± 0.04	(0.0004)
Esterified ferulates	0.45 ± 0.04	(<0.0001)	0.28 ± 0.04	(0.0003)	0.49 ± 0.08	(0.0004)
Etherified ferulates	−0.24 ± 0.06	(0.0034)	−0.39 ± 0.13	(0.0196)	−0.27 ± 0.11	(0.0323)
Residual	0.51 ± 0.06	(0.0001)	0.51 ± 0.09	(0.0010)	0.56 ± 0.08	(0.0002)

In smooth bromegrass, the basis for genetic variability in NDF concentration appears to be due, at least partially, to differential solubility of the cell wall at neutral pH (Casler and Hatfield, 2006). Low-NDF plants (i.e., those with low levels of etherified ferulates) appear have a cell wall that is less highly developed compared to the cell wall in high-NDF plants, even though divergent-NDF plants have similar total cell-wall concentrations (Casler and Hatfield, 2006). The important components of the cell wall are all present in low-NDF plants, but their greater solubility suggests a reduced state of structural development due to limited (delayed) development of covalent linkages with lignin, not necessarily due to delayed reproductive maturity. Because the ND-soluble portion of stem cell walls continues to increase with advancing maturity, at least up to the anthesis growth stage (Casler and Hatfield, 2006), there is little or no potential for low-NDF genotypes to "catch up" with high-NDF genotypes in terms of this delayed cell-wall development. The more ND-soluble portion of the cell wall is greater in low-NDF genotypes, and its tendency to increase with advancing maturity at the expense of the ND-insoluble fraction, magnifies these changes at more advanced stages of maturity. Arabinoxylans tend to become less highly branched with advancing maturity, measured by a decrease in the arabinose/xylan ratio (Morrison, 1974; Jung and Casler, 2006). Thus, reduced arabinoxylan branching and, by extension, reduced etherification of ferulates, may be the regulatory agent responsible for the increased solubility and fermentation at 24 h, creating a cell wall that is more open to attachment of rumen microorganisms and their enzymes to arabinoxylans. Retarded development of the cell wall at a given stage of reproductive maturity, particularly in stem tissue, may be responsible for the consistent and drastic reductions in fitness observed in smooth bromegrass and reed canarygrass populations selected for low NDF concentration (Casler, 1999a, 2005; Surprenant et al., 1988).

The remarkable similarity of results across all three species suggests that the roles played by lignin and etherfied ferulates in regulating digestion of the NDF fraction are fairly universal, at least within the temperate grasses. Our discussion has focused on genotypic variation for cell-wall components and its effects on genetic variation for digestibility of NDF in a very general sense, largely because all results were based on whole-plant tissue samples. In reality, much of the genetic variability for cell-wall components may arise from genetic variability for cell types that differ in extent and type of lignification. Such genetic variability is known to exist within multiple functional groups of plants, including a C3 grass (Ehlke and Casler, 1985), a C4 grass (Sarath et al., 2005), and a legume (Shenk and Elliott, 1971). Low-NDF/low-etherified ferulate genotypes may have a higher frequency of highly fermentable tissues made up of mesophyll, parenchyma, and/or chlorenchyma cells and a corresponding lower frequency of highly lignified tissues such as vascular bundles. The same is probably true for low-lignin genotypes, but the effects may be more localized to a more limited pool of tissues and/or cell types, as evidenced by the fact that reduced lignification per se does not generally negatively affect agronomic fitness (Casler, 2001), as occurs with genetically reduced NDF concentration.

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